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14. ABSTRACT
This project focuses on the novel finding from our group that the diaphanous-related formin protein DRF3 is a signaling molecule positioned downstream from the EGF receptor that intersects with the sine kinase Src in prostate cancer cells. Formins are effectors of small Rho-family GTPases like and provide a direct link between activated membrane receptors and the actin cytoskeleton. They also regulated by a large number of other activators including Src homology 3 (SH3)-containing adaptor proteins and Src family kinases, and can therefore serve as signal integrating platforms inside cell. Evidence was presented in the original proposal that the EGFR Drf3 Src signaling circuit appears to traverse cholesterol-rich "lipid raft" membranes in prostate cancer cells. Lipid rafts are choleterol- and sphingolipid-enriched membrane microdomains that serve as signal transduction platforms sequestering and excluding signaling proteins and by harboring pre-formed multi-protein complexes. have hypothesized in this project, and in our published work in this area, that cholesterol accumulation in prostate cancer cells may promote oncogenesis by altering the nature of—and/or the of—signals that flow through lipid raft microdomains. Several new lines of evidence consistent this hypothesis have been produced in year 2 and are described and summarized in this report.

15. SUBJECT TERMS
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"DRF3 as a Cholesterol-dependent Regulator of Src in Prostate Cancer"
Grant number: A81XWH-06-1-0197
Progress report for 12/14/2006- 12/13/2007

INTRODUCTION

This project focuses on the novel finding from our group that the diaphanous-related formin protein DRF3 is a signaling molecule positioned downstream from the EGF receptor that intersects with the tyrosine kinase Src in prostate cancer cells. Formins are effectors of small Rho-family GTPases like CDC42 and provide a direct link between activated membrane receptors and the actin cytoskeleton. They are also regulated by a large number of other activators including Src homology 3 (SH3)-containing adaptor proteins and Src family kinases, and can therefore serve as signal integrating platforms inside the cell. Evidence was presented in the original proposal that the EGFR→Drf3→Src signaling circuit appears to traverse cholesterol-rich "lipid raft" membranes in prostate cancer cells. Lipid rafts are cholesterol- and sphingolipid-enriched membrane microdomains that serve as signal transduction platforms by sequestering and excluding signaling proteins and by harboring pre-formed multi-protein complexes. We have hypothesized in this project, and in our published work in this area, that cholesterol accumulation in prostate cancer cells may promote oncogenesis by altering the nature of—and/or the types of—signals that flow through lipid raft microdomains.

BODY

In year 2, we have completed Task 2, made significant progress on Tasks 1 and 3 and on preliminary studies for Task 4.

Task 1. Determine the biological consequences of increasing and lowering cellular DRF3 levels by overexpression or gene silencing experiments in prostate cancer cells.

HeLa and LNCaP cells were transiently transfected with a control pool of non-targeting siRNA or with siRNA targeting the ORF of the DRF3 gene. Isolation of total mRNA was followed by semi-quantitative RT-PCR and clearly demonstrated efficient depletion of DRF3 mRNA by RNAi. Successful knockdown of the DRF3 mRNA was also shown in prostate cancer cell lines on the protein level with a specific antibody to DRF3.

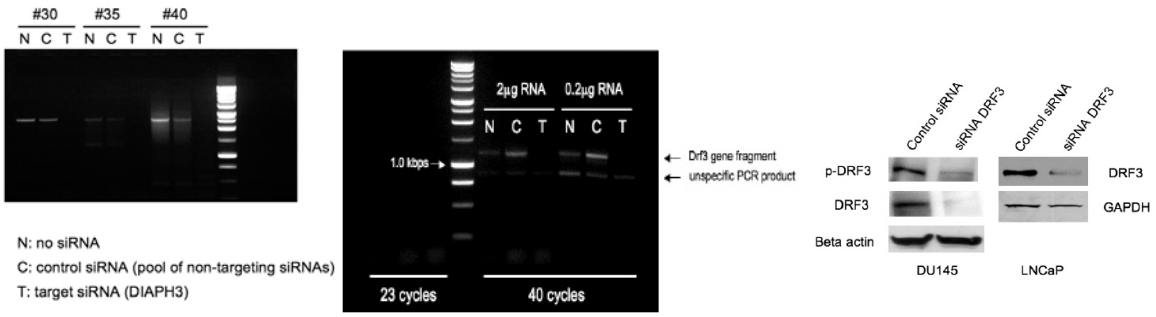


Figure 1: Semi-quantitative RT-PCR with two independent primer sets shows specific depletion of the DRF3 mRNA (T) but no effect of the control siRNA pool (C). Note that the intensity of the unspecific PCR product is not affected by either siRNA. Western Blot analysis confirmed the RT-PCR data and shows successful reduction of the DRF3 protein with a specific commercial antibody as well as our phosphosite-specific antibody.

The biological consequences of silencing the DRF3 gene expression in prostate cancer cells was then further explored in cell invasion assays, cell adhesion assays and colony formation assays. Reduction of DRF3 levels dramatically increased the invasive behavior of LNCaP prostate cancer cells, which was also mirrored by their ability to form colonies in soft agar. Cell adhesion in these cells was slightly reduced and an effect on cell proliferation rate could not be observed. Another strong phenotype was observed in DU145 prostate cancer cells. Here, knockdown of DRF3 resulted in increased cell membrane blebbing, a phenomenon that is attributed to a “softening” of the actin cytoskeleton scaffold in response to receptor tyrosine kinase activation. These findings are in good agreement with the general notion that formins play a pivotal role in actin polymerization and that remodeling of the actin cytoskeleton is important for motility and chemotaxis of cancer cells. Consequently, we show for the first time that the lipid-raft protein DRF3 is involved in modulating cell-cell and cell-matrix adhesions with concomitant increase in cell motility.

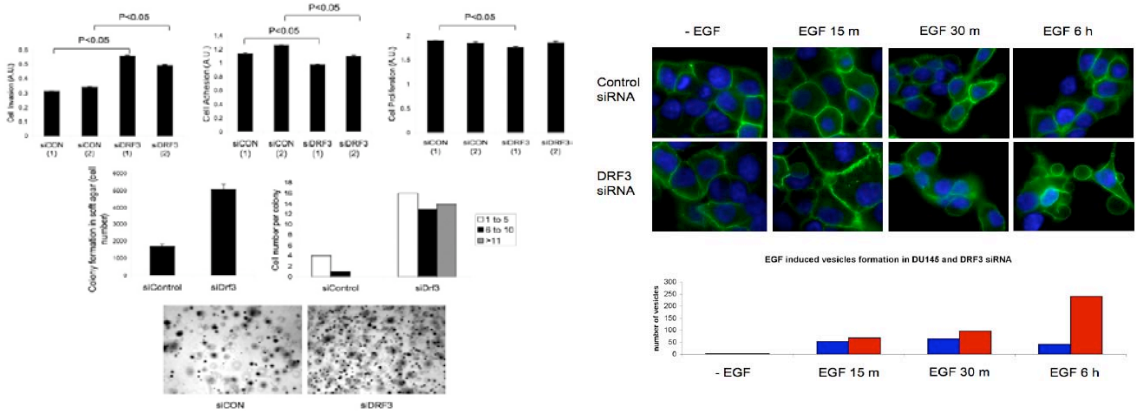


Figure 2: Knockdown of DRF3 by RNAi changes the motility of LNCaP and DU145 prostate cancer cells. Left: In the virtual absence of DRF3, LNCaP cells dispay enhanced invasion in matrigel chamber assays, decreased adhesion and anchorage independent growth, whereas cell proliferation rates are unaffected. Right: Dynamic membrane blebs in DU145 prostate cancer cells. Treatment of DU145 cells with EGF results in a rounded mode of cellular invasion, which is strongly increased in the absence of the DRF3 protein. A quantification of the number of observed membrane blebs on the surface of DU145 cells clearly demonstrates that blebs occur more frequently on cells with silenced DRF3-expression (red) in comparison to wild-type DU145 cells transfected with control siRNA (blue).

As further proposed in Task1, a constitutively active variant of DRF3 was generated by deletion of 263 amino acids in the N-terminus, comprising the entire GTPase-binding domain (GBD) of DRF3. Expression of the constitutively active DRF3 (ISO847) in COS7 cells resulted in a signal at the expected molecular size of 97kDa in comparison to the 127kDa of the full-length DRF3 protein.

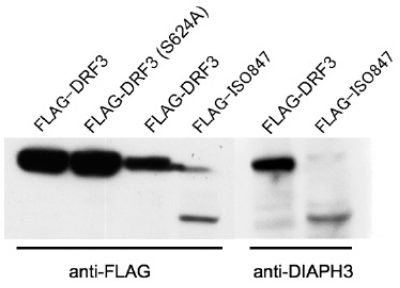


Figure 3: Deletion of the GTPase-binding domain of DRF3. Western blotting with a FLAG-specific antibody shows expression of FLAG-tagged full-length DRF3 (127kDa) and the deletion mutant ISO847 (97kDa), which lacks the GTPase-binding domain (GBD). An identical pattern can be seen with an anti-DRF3-specific antibody.

Together with the previously generated S624A phosphorylation mutant of DRF3, we tested the consequences of these sequence alterations on DRF3 activity and its ability to bind companion proteins. Since the primary focus of the proposal is on the interaction of DRF3 and the Src tyrosine kinase, we decided to explore how serine to alanine exchange at position 624 of the DRF3 protein would affect its interaction with Src. To this end, COS7 cells were transfected with FLAG-tagged DRF3 in combination with either wild-type Src or a constitutively activated form of Src (Y529F). Co-immunoprecipitation of the tagged DRF3 protein and analysis of Src binding revealed that the DRF3-Src interaction could be modulated by EGF. In the presence of EGF, binding of endogenous wild-type Src to DRF3 was enhanced, whereas constitutively active Src (Y529F) displayed decreased binding to DRF3 after EGF stimulation.

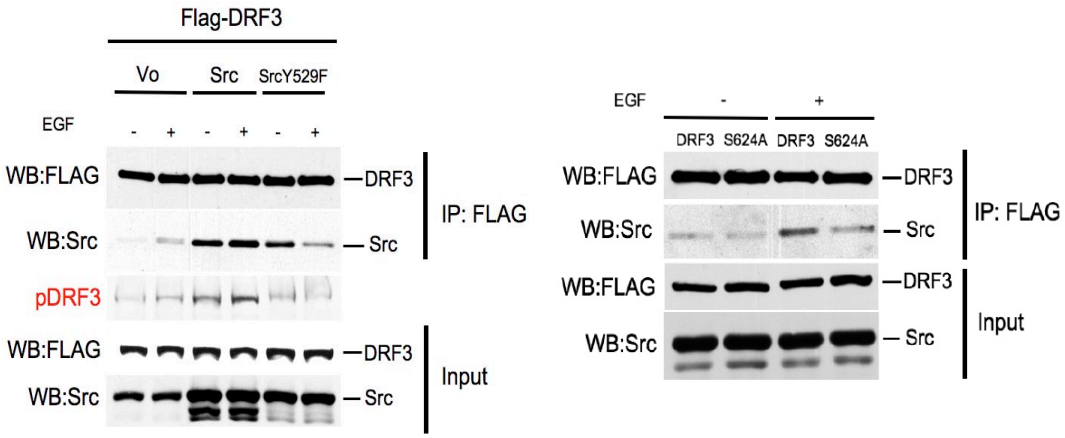


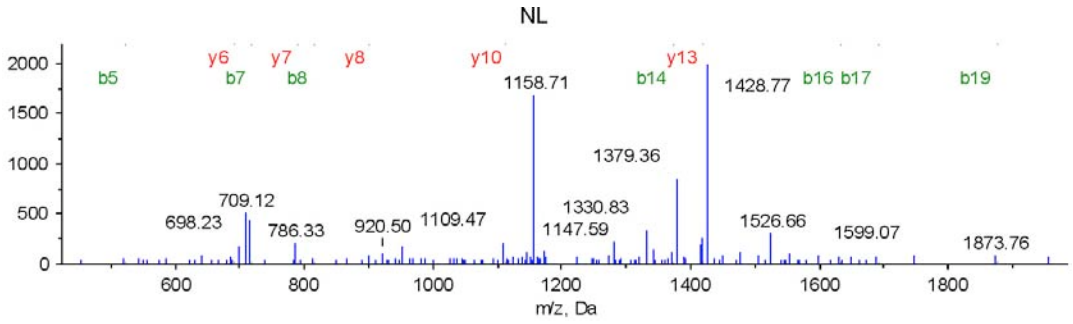
Figure 4: EGF modulates binding of Src to DRF3 via serine 624. FLAG-tagged DRF3 was expressed in COS7 cells together with wild-type (Src) or activated (Y529F) Src tyrosine kinase. Immunoprecipitation of DRF3 with anti-FLAG antibodies and detection of co-immunoprecipitated proteins reveals that stimulation with EGF increases binding of endogenous Src but not activated Src to DRF3. Right: Phosphorylation of serine 624 in response to EGF is required for Src binding to DRF3. Binding of Src to DRF3 is enhanced in response to EGF only if the phosphorylation site at serine 624 is present. A serine to alanine amino acid substitution at this position abolished the EGF effect.

Two possible scenarios could provide an explanation for these observations: Activation of endogenous wild-type Src by EGF increases its affinity to the DRF3 protein, whereas constitutively activated Src (Y529F) is already in its activated conformation and binding cannot be enhanced by EGF treatment. However, the obvious decrease in Src (Y527F) binding after activation with EGF is difficult to explain. In an alternative scenario, a phosphorylation event at DRF3 would provide a docking site for wild-type Src. In order to test the hypothesis that a phosphorylation event on a DRF3 residue modulates Src binding, we monitored the phosphorylation status of DRF3 at position 624 with our specific phospho-antibody. Interestingly, the phosphorylation pattern of DRF3 at position 624 correlates with Src binding to DRF3. This observation prompted us to repeat the co-immunoprecipitation experiment with the S624A phosphorylation mutant of DRF3 to further pinpoint the exact phosphorylation event. As shown in figure 4, binding of Src to wild-type DRF3 is enhanced after treatment with EGF. This effect, however, is abolished by exchanging serine at position 624 to alanine, further supporting the view that a phosphorylation event at this site provides a binding motif for the Src kinase. Binding of Src to the mouse homolog of DRF3 (mDia2) has been reported but a modulation of Src binding by EGF treatment and the involvement of a phosphorylation site at serine 624 are en-

tirely novel observations that will help to elucidate how these two molecules are regulated in prostate cancer.

Task 2. **(i)** Identify the phosphorylation sites on Drf3 that are regulated by EGFR activation and **(ii)** determine the functional consequences of phosphorylation at these sites.

We now have overwhelming evidence that DRF3 is phosphorylated at serine 624 in response to EGFR activation. Since submission of the last progress report, the phosphorylation event has been demonstrated by mass spectrometry in a series of independent experiments. Recently, we have been revisiting the mass spectrometry approach to gather preliminary data that will serve to *quantify* the phosphorylation status of DRF3 by stable isotope labeling with amino acids in cell culture (SILAC). In these preliminary studies, we were again able to confirm phosphorylation of DRF3 at serine 624. In unstimulated LNCaP prostate cancer cells that were harvested under steady state conditions, the majority of identified DRF3 molecules were not phosphorylated at serine 624. However, a smaller number of phosphopeptides was identified, demonstrating that under serum-containing growth conditions the pool of DRF3 molecules is partly phosphorylated. Quantification of the typical ratios between phosphorylated and unphosphoylated DRF3 proteins by SILAC will give us more insight into the dynamics of this phosphorylation event in unstimulated and growth-stimulated prostate cancer cells.



Con	Sequence	Modification	Delta m/z	mass
99	MPFSGPVPPPPPLGFLGGQNSPPL PILPFGLKPK	Deamidated(N)@20; Phospho(S)@21	-0.1472	3570.7283
99	MPFSGPVPPPPPLGFLGGQNSPPLPILPFGLKPK		1.153	3491.0783
99	MPFSGPVPPPPPLGFLGGQNSPPLPILPFGLKPK		0.433	3490.3583
99	MPFSGPVPPPPPLGFLGGQNSPPLPILPFGLKPK	Oxidation(M)@1	1.4482	3507.3683
99	MPFSGPVPPPPPLGFLGGQNSPPLPILPFGLKPK	Oxidation(M)@1	-0.0218	3505.8983

Figure 5: The pool of DRF3 molecules is partly phosphorylated under steady state conditions. LNCaP cells were transfected with plasmid DNA encoding FLAG-tagged DRF3. Protein purification with anti-FLAG M2-agarose beads and subsequent mass spectrometrical analysis revealed peptide fragments with both phosphorylated and unphosphorylated serines corresponding to position 624 in the DRF3 protein.

As has been described in the previous report, we have generated a custom-made phosphosite-specific antibody in rabbits using a synthetic phosphopeptide comprising the DRF3 phosphorylation site around serine 624. We have now thoroughly vetted this antibody and are able to show conclusively that the antibody recognizes the phosphorylation site with a high degree of specificity in various cell backgrounds.

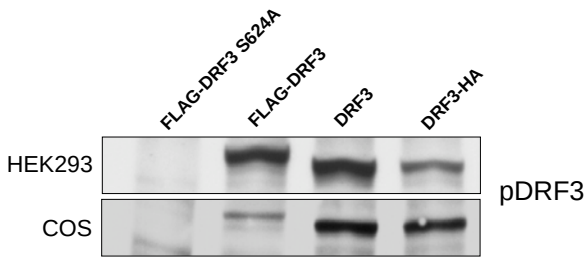


Figure 6: Detection of phospho-serine 624 with a custom-made antibody. HEK293 and COS7 cells were transfected with wild-type DRF3 or the S624A mutant and grown in serum-containing medium. Western-Blot analysis demonstrates that the phosphosite-specific antibody binds to untagged or tagged DRF3 protein but has no affinity to the phosphosite-mutant of DRF3, in which the phosphorylation site at serine 624 has been altered to alanine.

Task 3. **(i)** Identify the binding partners of DRF3 and the physiological contexts in which they act.

Based on our novel finding that the DRF3 protein is phosphorylated in response to EGF, we are now exploring the physiological consequences of this phosphorylation event and how it impacts on DRF3 interaction partners. The DRF3 protein is an important actin regulatory molecule that responds to multiple stimuli. In the absence of activators, DRF3 is believed to exist in an autoinhibited conformation, where its GTPase binding domain (GDB) interacts with its C-terminal autoinhibitory domain. As a result the socalled FH2 domain, which is required for actin nucleation, is sequestered. Rho family GTPases like Rho A or CDC42 bind to and activate the DRF3 molecule by exposing the FH2 domain. This activation process is believed to be highly cooperative, with DRF3 serving as a signaling platform that integrates various input signals. The mechanisms of cooperativity, however, are not well understood. Therefore, we set out to explore how activation of DRF3 by GTPases cooperates with the phosphorylation event at serine 624. Of the various interaction partners that are able to bind to the DRF3 molecule, we focused on the FH1 domain, which harbors the phosphorylation site at serine 624. One important interaction partner of DRF3 is Profilin-1, which serves as a G-actin transport molecule in the cell. It binds with high affinity to the FH1 domain of DRF3 and delivers actin in its unpolymerized form to the FH2 domain, where it disassociates and allows actin to polymerize. Against the backdrop of these reflections, we wanted to address the question whether phosphorylation of an activated DRF3 molecule could serve as an additional control step in the regulation of actin polymerization. To this end, COS7 cells were transfected with either wild-type DRF3 or its mutated form S624A. In order to activate both molecules, the transfection was supplemented with a constitutively activated form of CDC42, which binds to the GBD of DRF3 and renders this formin biologically active. Additionally, the earlier described GBD deletion mutant of DRF3 (F1F2+C) was included in these experiments, which consists of the FH1 domain, the FH2 domain and the C-terminus but lacks the GBD. The purpose of including this last construct was to test a constitutively active version of DRF3 that would not require the presence of activated CDC42. Surprisingly, co-immunoprecipitation of the FLAG-tagged, constitutively active version of DRF3 (F1F2+C) revealed that Profilin-1 was bound to the FH1 domain and stimulation with EGF completely abolished the interaction. This result points to an interesting mechanism that employs phosphorylation to control binding of Profilin-1 to DRF3 and ultimately actin polymerization. Binding of Profilin-1 to the DRF3 phospho-mutant was enhanced in the presence of EGF and no binding of Profilin-1 to wild-type DRF3 could be detected. Currently we are trying to further explore why Profilin-1 (1) is not pre-

sent in the immunoprecipitate of wild-type DRF3 and (2) shows weak binding to the DRF3 phosphorylation mutant.

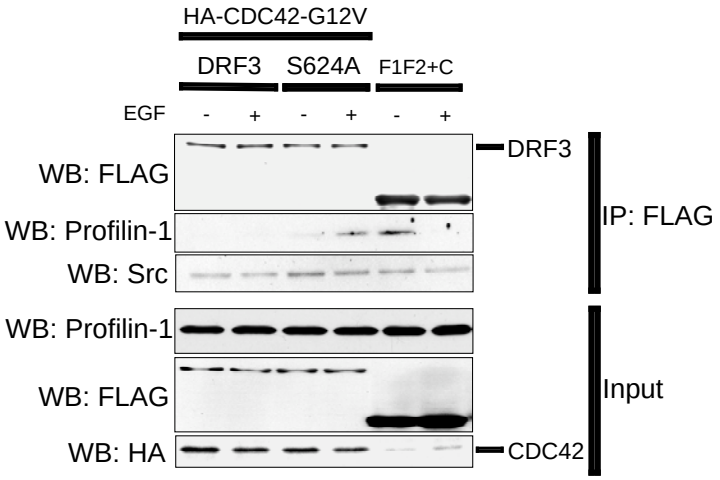


Figure 7: EGF-treatment interferes with binding of Profilin-1 to activated DRF3. COS7 cells were transfected with either FLAG-tagged wilde-type DRF3 or FLAG-tagged phospho-mutant S624A in addition to constitutively activated CDC42-G12V, which served as an activator of DRF3/S624A. Alternatively, a constitutively active form of DRF3 (F1F2+C) was tested in this experiment that does not require activation by CDC42. Immunoprecipitation of expressed FLAG-tagged F1F2+C demonstrates that Profilin-1 interacts with the constitutively active form of this formin (WB: Profilin-1). However, stimulation of the cells with EGF completely abolishes this interaction.

Task 4. **(i)** Determine whether DRF3 expression functionally suppresses or otherwise alters Src activity or subcellular transit. **(ii)** Determine the subcellular localization and physiological contexts where Src and DRF3 interact and their relevant inteacting partners. **(iii)** Determine whether DRF3 activities are dependent on the presence of Src.

Src-transformed NIH3T3 fibroblasts have been used as a model system by other groups to study the physiological responses of cells when activated by a strong oncogene. We decided to create NIH3T3 cells that express wild-type and constitutively active versions of the Src kinase to test whether expression of wild-type or mutant DRF3 would suppress or enhance the phenotype of Src-transformed NIH3T3 cells. To this end, constructs expressing wild-type Src and Src (527F) were cloned into the retroviral vector pLNCX and transfected into HEK293 T cells for virus production. Retroviruses were collected and used for infection of NIH3T3 cells. After selection of geneticin-resistant cells, stable transformed clones were isolated and are now being further characterized. Interestingly, expression of high levels of the Src oncogene are not sufficient to transform NIH3T3 cells as no morphological changes could be observed. However, expression of constitutively activated Src (Y527F) results in a dramatic transition from the flat, fibroblastic morphology to the characteristic transformed phenotype with round-up morphology. The Src-transformed NIH3T3 cells will serve in future experiments as a model to gain further insight into how Src and DRF3 signaling pathways intersect. Currently, these cells are being transfected with our collection of DRF3 variants to test our hypothesis that the EGF receptor is signaling through DRF3

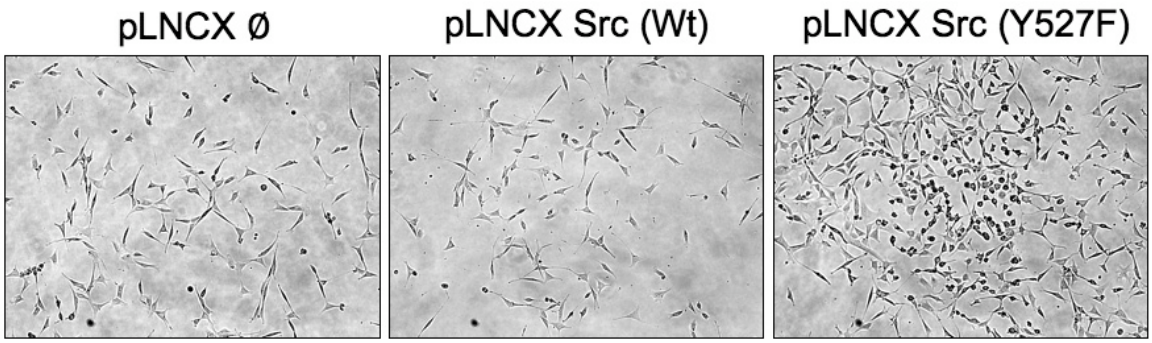


Figure 8: Transformation of murine NIH3T3 fibroblasts with retroviral vectors encoding wild-type Src or activated SrcY527F. The SrcY527F-transformed cells are not only morphologically transformed in comparison to cells harboring wild-type Src, but are also highly invasive.

KEY RESEARCH ACCOMPLISHMENTS

- In year 2 we have demonstrated that knockdown of DRF3 enhances cell motility and invasion of prostate cancer cells
- We have shown that the non-receptor tyrosine kinase Src interacts with the DRF3 molecule in response to EGF and phosphorylation of serine 624 is necessary for this interaction.
- We provide preliminary data, which suggest that phosphorylation of serine 624 abolishes binding of profilin/actin to DRF3. Consequently, phosphorylation of serine 624 could have direct implications for the regulation of actin-polymerization by DRF3.

REPORTABLE OUTCOMES

To date, three publications attributable in whole or part to funding from this grant have been published.

Hager, M.H., Solomon, K.R., and **Freeman, M.R.** (2006) The role of cholesterol in prostate cancer. Current Opinion in Clinical Nutrition and Metabolic Care 9:379-385.

Lutchman, M., Solomon, K.R., and **Freeman, M.R**. Cholesterol, cell signaling and prostate cancer. In: Prostate Cancer: Novel Biology, Genetics and Therapy. Chung, L.W.K., Isaacs, W.B., and Simons, J.W. Second edition. pp. 119-137. Humana Press, Totowa, NJ, 2007.

Freeman, M.R., Cinar, B., Kim, J., Mukhopadhyay, N.K., Di Vizio, D., Adam, R.M., and Solomon, K.R. (2007) Transit of hormonal and EGF receptor-dependent signals through cholesterol-rich membranes. Steroids 72:210-217.